Neuroimmune Response Activation by The Toll-Like Receptor 7 Agonist Imiquimod Increases Alcohol Self-Administration and Gene Expression in Rats

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Abstract

Men and women world-wide suffer from alcohol use disorder (AUD), defined as a chronic brain disease characterized by the loss of an individual's control over their alcohol intake. Emerging evidence has found that AUD is likely linked to specific neuroimmune responses in the brain. Neuroimmune responses can be triggered by numerous events such as infection, trauma, and stress, and they result in the increased expression of various proinflammatory molecules and receptor proteins. The purpose of this research was to further understand the relationship between the neuroimmune cascade triggered by activation of the toll-like receptor-7 protein (TLR7) that canonically detects viral single-stranded RNA, focusing on its effect on ethanol drinking behavior. Male and female Long Evans rats were trained to self-administer 15% ethanol solution in operant chambers and their drinking patterns were tracked during daily 30-minute sessions. Every 15 days, 10 mg/kg of imiquimod, a specific TLR7 agonist, was injected intraperitoneally in the male and female rats in order to induce a neuroimmune cascade. Increased alcohol self-administration was observed the day following imiquimod injections for both the male and female groups; however, there were some differences in the expression of the behavior. In females, this effect was seen after the first injection while the male group did not show this increase until the third injection suggesting a difference in TLR7 sensitization between sexes. Gene expression data was analyzed via real-time RTPCR from the nucleus accumbens core, a region in the brain implicated in drug addiction and reward in order to test for expression of TLR7 as well as TLR3, a receptor that detects double-stranded RNA. Ultimately, further testing may provide insight necessary in order to understand which specific factors of this neuroimmune cascade are responsible for the phenomena observed. Conclusively, understanding which specific neurobiological factors of neuroimmune activation contribute to the pathology of AUD is necessary for developing improved treatment options.

INTRODUCTION:

Alcohol use disorder (AUD) is characterized as a brain disease associated with a loss of control over alcohol intake. Our research focuses on understanding the link between AUD and the body's innate immune system due to emerging evidence that AUD is likely linked to specific neuroimmune responses activated by the body's innate immune system (Crews et. al. 2011). Neuroimmune responses can be triggered by numerous events such as infection, trauma, or stress and they result in an increase in the expression of various proinflammatory molecules and receptor proteins that can have lasting effects on the neurons associated with the brain's reward system (Crews et. al., 2017a; Crews and Vetreno, 2016). Toll-like receptor proteins (TLRs) are a class of pattern-recognition receptors (PRRs) utilized by the innate immune system that detect molecular signatures known as pathogen-associated molecular patterns (PAMPS) or damage-associated molecular patterns (DAMPS). Situated on microglia in the brain, TLR activation induces a neuroimmune cascade that can result in changes in the neurocircuitry and gene expression in the brain (Okun et al. 2011). Recent studies have shown an essential relationship between their activation and addiction-like behavior (Crews et al. 2017a). In human studies, the post-mortem brains of individuals with a history of AUD showed increased expression levels of TLRs (Crews et al. 2017b). Previously our lab has found that activating TLR3, which normally detects double-stranded RNA, in Long Evans rats via the agonist polyinosinic: polycytidylic (poly (I:C)) resulted in significantly increased alcohol self-administration (Randall et. al., 2019). Interestingly, the activation of TLR3 resulted in increased expression of TLR7, a related receptor that typically detects single-stranded RNA. The increased expression of TLR7 in response to TLR3 activation catalyzed our interest in targeting TLR7 in order to observe its effects on operant alcohol self-administration in rats.

TLR7 is encoded on the X chromosome and also escapes X chromosome inactivation, presumably making it more highly expressed in female immune cells than in males (Souyris et. al., 2018). Evidence suggests that there are sex-specific differences in TLR immune activation; specifically, females tend to show increased activation of proinflammatory responses compared to their male counterparts (Klein and Flanagan 2016). We were interested in understanding how activating TLR7 via agonist imiquimod would affect alcohol self-administration, and how the neuroimmune cascade induced by the same dose of imiquimod would differ between sex and if this would have any implications on alcohol self-administration.

Here, we administered four 10 mg/kg doses of imiquimod once every 15 days to male and female adult Long Evans rats and then monitored alcohol self-administration through daily operant chamber sessions. After the fourth imiquimod injection, gene expression data was analyzed using real-time RTPCR on the nucleus accumbens core (AcbC), a brain region implicated in modulating reward and drug addiction in order to analyze expression of the TLRs. We found that activation of TLR7 via agonist imiquimod increased alcohol self-administration in both males and females. In females we observed increased alcohol self-administration after the initial imiquimod injection while in males we observed increased alcohol self-administration after repeated imiquimod injections. These experiments seek to further the understanding of how neuroimmune activation, specifically via TLR7, impacts alcohol self-administration. Ultimately, understanding the neurobiological factors that contribute to AUD is important for the development of enhanced and more specific treatments.

METHODS:

Animals

Adult male (n=24) and female (n=24) Long Evans rats (Envigo-Harlan, Indianapolis, IN) arrived at 7 weeks old and were handled daily for 1 week prior to the start of the experiment. All rats were doubled housed in ventilated cages in same-sex pairs. Rats had ad-libitum access to food and water in the home cage. The rats were kept in a temperature and humidity-controlled colony room that ran on a 12-hour light/dark cycle (lights on at 07:00). All experiments were conducted during the light cycle. Animals were under the care of the veterinary staff from the Division of Comparative Medicine at UNC-Chapel Hill. All of the procedures followed the guidelines established by the NIH Guide to Care and Use of Laboratory Animals and institutional guidelines.

Chambers/Apparatus

Self- administration chambers (Med Associates, Fairfax, VT) were used in order to test for changes in drinking patterns and to track alcohol consumption and locomotor rate. Self-administration sessions that lasted 30 minutes were held each day Monday through Friday for each individual rat. The chambers had two levers on opposing sides of the chambers, with a corresponding cue light above each lever. The levers were on a fixed-ratio 2 reinforcement schedule (FR-2), meaning two lever presses resulted in one reinforcement. Upon pressing the left lever twice, the corresponding cue light was illuminated and a tone was turned on as 0.1 mL of ethanol (EtOH) solution was delivered into the receptacle on the left side. Upon pressing the right lever, which is an inactive lever, there was no programmed consequence. The chambers were individually located within a larger sound-attenuating cubicle containing a fan that was

meant to circulate air and mask outside noise. The chambers were also equipped with infrared photo-beams in order to track locomotor activity during a given session. During a self-administration session, the amount of ethanol administered, the number of times each lever was pressed, locomotor rate, and number of head-pokes into the left and right receptacle were recorded.

Ethanol Self-Administration Training

Rats first underwent a 16-hour overnight session with 10% sucrose 2% alcohol (w/v; 10S/2A) in order to learn the association between pressing the alcohol active lever and receiving alcohol in the left receptacle. Following this session, rats underwent a sucrose fading procedure as previously described (Besheer et al., 2010). Briefly, rats trained on daily 30-minute sessions in which the sucrose concentration slowly decreased and the alcohol concentration increased in the following order: 10S/2A, 10S/5A, 10S/10A, 5S/10A, 5S/15A, 15A, 20A, 15A. Following the sucrose fading, 15% ethanol (v/v) served as the final and maintenance condition for daily self-administration sessions over the next 24 days.

Imiquimod Injections

Imiquimod (batch 0000025236, Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 45% hydroxypropyl-beta-cyclodextrin (Acros Organics, Geel, Belgium). Every 15 days, a 10 mg/kg dose of imiquimod was administered intraperitoneally (IP) while control rats received equivalent injections of hydroxypropyl-beta-cyclodextrin.

Tissue Collection and Real Time RTPCR

Rats were anesthetized with carbon dioxide (CO_2) (Airgas, Radnor, PA) and then brains and spleens were collected 24 hours after the 4th injection. Spleens were weighed and adjusted according to body weight. RT-PCR (reverse transcription [RT] followed by real-time polymerase chain reaction [PCR]) was conducted to quantify levels of mRNA of target neuroimmune genes in the nucleus accumbens core (AcbC). Total mRNA was extracted and reverse transcribed as previously described (Vetreno and Crews, 2012; Vetreno et al., 2013). Real-time PCR using SYBR green PCR Master Mix (Life Technologies, Carlsbad, CA) was run with an initial activation for 10 min at 95 C, followed by 40 cycles of denaturation (95 C, 15s), annealing/extension (57-58 C, 1 min), and finally a melt curve. The primer sequences are presented in Table 1. The threshold cycle (CT) of each target product was determined and the ΔΔCT method was used to calculate the percent change relative to control (CON).

Table 1:

List of Primers for RTPCR		
TLR3	Forward:	5' –GCA ACA ACA ACA TAG CCA AC-3'
	Reverse:	5' –CCT TCA GGA AAT TAA CGG GAC-3'
TLR7	Forward:	5' –AGC TCT GTT CTC CTC CAC CA-3'
	Reverse:	5' –CAT GGG TGT TTG TGC TAT CG-3'

Statistics

Data are expressed as mean ± SEM. To analyze behavioral data, unpaired t-tests were used to compare the ethanol lever responses, ethanol intake (g/kg; calculated from the number of reinforcers received) and locomotor activity on the day of imiquimod injection and the day after versus control rats. Real-time PCR results were assessed using unpaired t-tests to compare gene expression of target mRNA in imiquimod rats versus control rats within sex. Unpaired t-tests

were used to compare spleen weight adjusted to body weight of imiquimod rats versus control rats. Statistical significance was declared at $p<0.05$.

Experiments:

Experiment 1 - Effects of imiquimod on ethanol self-administration in male rats

Every 15 days, self-administration trained male Long Evans rats were injected with imiquimod (0 or 10 mg/kg, IP) 2 hours prior to a standard 30-minute self-administration session (n=12 control, n=12 imiquimod). Daily self-administration was resumed the following day for 15 days. Four rounds of imiquimod and control injections were given in total over a 46-day span. To examine the effects of imiquimod on expression of TLR7 and TLR3 in the AcbC, animals were sacrificed 24 hours after the 4th round of imiquimod injections for tissue and organ collection.

Experiment 2 - Effects of imiquimod on ethanol self-administration in female rats

The exact experimental design of Experiment 1 was replicated with 24 female rats (n=12 control, n=12 imiquimod) in order to observe any sex-specific differences.

RESULTS:

Repeated TLR7 activation via imiquimod increased alcohol self-administration in males

An increasing trend in alcohol self-administration was observed the day after each subsequent imiquimod injection in the male rats, with the greatest increase in alcohol self-administration 24 hours after the third injection (Fig.1A/Fig.2A). After the second injection there is a strong increasing trend ($p= 0.058$) in ethanol intake (grams of ethanol consumed/kg of body weight) observed in the imiquimod group. Intake was significantly ($t(22)=2.44$, $p< 0.05$) higher in the

imiquimod group 24 hours after the third injection (Fig.1A). Alcohol lever presses appear to increase in the imiquimod group the day after the second injection and are significantly ($t(22)= 2.22$, $p< 0.05$) higher the day after the third injection (Fig.2A). Two hours after injections two through four, rats in the male imiquimod group had significantly reduced ethanol intake (2^{nd} injection: $t(22)= 3.85$, $p<0.05$; 3^{rd} injection: $t(22)= 6.59$, $p<0.05$; 4^{th} injection: $t(22)= 5.02$, $p<0.05$) as well as alcohol lever presses (2^{nd} injection: $t(22)= 3.96$, $p<0.05$; 3^{rd} injection: $t(22)= 7.3$, $p<0.05$; 4^{th} injection: $t(22)= 5.31$, $p<0.05$). (Fig. 1A/Fig.2A).

Initial TLR7 activation via imiquimod increased alcohol self-administration in females

A significant increase in alcohol self-administration was observed in the female imiquimod group the day following the first injection (Fig.1/Fig.2). Ethanol intake (g/kg) was significantly; higher in the female imiquimod group 24 hours after the first injection ($t(22)=2.56$, $p< 0.05$ and third injection ($t(22)=2.088$, $p<0.05$; Fig.1B). Alcohol lever presses were significantly higher in the female imiquimod group 24 hours after the first injection ($t(22)= 2.17$, $p< 0.05$) and there is a trend for an increase in alcohol lever presses 24 hours after the third injection ($p=0.087$; Fig.2B). Female imiquimod rats had reduced ethanol intake as well as alcohol lever presses on the days of imiquimod injections one through three (Fig.1B/Fig.2B); however, ethanol intake ($t(22)=3.71$, $p<0.05$) and alcohol lever presses ($t(22)=3.90$, $p<0.05$) were only significantly reduced on the day of the third injection.

Reduced locomotion and weight loss suggest imiquimod induced an immune response in both sexes

Imiquimod is a known agonist of the toll-like receptor 7 protein; however, in order to determine whether a 10 mg/kg dose of imiquimod was sufficient to activate a significant immune response

several variables were tested. Some of the main symptoms of a stereotyped sickness response are reduced activity and weight loss, two variables we tracked for daily changes. As a general trend, indications of a sickness response were observed as soon as two hours after imiquimod injections that affected the imiquimod groups behaviorally during the self-administration sessions on the day of injections. The locomotor rates of the male and female imiquimod groups were generally significantly lower on days of injections (Fig.3); however, there were some differences in the patterns observed. The locomotor rate of the male imiquimod group was not affected by the first injection. It wasn't until the second imiquimod injection that the locomotor rates of the male imiquimod group were significantly lower than the control group ($t(22)=3.30$, $p<0.05$), and this trend was consistent with each subsequent imiquimod injection (third injection: $t(22)=2.43$, $p<0.05$; fourth injection: $t(22)=4.54$, $p<0.05$) (Fig.3A). Comparatively, the locomotor rate of the female imiquimod group was significantly lower than the control group after the initial imiquimod injection ($t(22)=2.84$, $p<0.05$) and with each subsequent injection a similar trend was observed (2^{nd} injection: $p=0.056$; 3^{rd} injection: $t(22)=3.83$, $p<0.05$; 4^{th} injection: $p=0.061$) (Fig.3B).

The weights of both male and female rats in the imiquimod groups showed a decreasing trend the day following injections (Fig.4) and then resumed normal weight gain until the subsequent injection.

Since spleens are a storage site for immune cells and enlarged spleens, termed splenomegaly, are indicative of a hyperactive immune system, they were collected and weighed 24 hours after the fourth imiquimod injection in order to assess whether an immune response had been activated in the imiquimod groups. Spleen weights were adjusted to account for differences in body weight. Both male and female rats in the imiquimod group had significantly heavier

spleens compared to the control group ($t(22)=7.51$, $p<0.05$; $t(22)=5.78$, $p<0.05$, respectively) (Fig.5).

Repeated activation of TLR7 increases gene expression of TLRs in males and females

In order to determine how the TLR7 agonist imiquimod alters toll-like receptor gene expression in rat AcbC, real-time RTPCR was utilized in order to determine expression of TLR7 and TLR3 in the imiquimod group compared to the control group. Male imiquimod rats had significantly increased expression of TLR3 in the AcbC 24 hours after the fourth injection compared to the control group ($t(20)=3.22$, $p<0.05$) (Fig.6A). Similarly, female imiquimod rats also had significantly increased expression of TLR-3 in the AcbC 24 hours after the fourth injection compared to the control group ($t(22)=2.20$, $p<0.05$) (Fig.6B). While there was increased expression of TLR3 in both males and females, only imiquimod females had increased expression of TLR7 in the AcbC 24 hours after the fourth injection compared to the control group ($t(22)=4.73$, $p<0.05$) (Fig.7B). There was not increased expression of TLR7 in the AcbC of male imiquimod rats after the fourth injection (Fig.7A).

DISCUSSION:

In this study, we evaluated the effects of the neuroimmune cascade triggered by TLR7 activation via agonist imiquimod on operant ethanol self-administration including mRNA levels of the neuroimmune genes TLR3 and TLR7 in the AcbC. Our results indicate that activation of TLR7 via imiquimod increased operant ethanol self-administration, induced an immune response, and increased gene expression of the neuroimmune genes TLR3 and TLR7.

Both male and female rats in the imiquimod group showed increases in ethanol intake and alcohol lever presses 24 hours after imiquimod injections; however, this increase was observed in the female imiquimod group after the first injection while imiquimod males showed this increase after repeated injections. Imiquimod females had significantly increased alcohol self-administration 24 hours after the first and third injections, which remained elevated for another day before dropping back to baseline drinking levels (Fig.1B/Fig.2B). An important consideration for the ethanol self-administration data is that rats experienced weight loss after injection days, affecting the ethanol intake (g/kg) calculated for that day. This may explain why ethanol intake (g/kg) after the third injection is significantly higher for imiquimod females while alcohol lever presses after the third injection are not considered significant despite showing a trend for an increase in alcohol lever presses. Alcohol self-administration did not increase after the second imiquimod injection, which could potentially be attributed to a short-term change in sensitization of the TLR7 receptor after the initial injection. Following a similar trend, overall changes in drinking behavior were observed after the initial imiquimod injection. Imiquimod females drank less on injection days starting after the first injection and continuing after the second and third injections (Fig.1B/Fig.2B). There was no decrease in alcohol self-administration on the day of the fourth injection, again potentially indicating a short-term change in sensitization of the TLR7 receptor.

Comparatively, imiquimod males did not show changes in drinking behavior after the initial imiquimod injection. Starting with the second injection, alcohol lever presses as well as ethanol intake (g/kg) increased sequentially with each injection with the greatest increase in alcohol self-administration occurring 24 hours after the third injection. Likewise, male imiquimod rats drank less on the days of injections two through four. This increase in alcohol

self-administration along with each injection indicates that repeated TLR7 activation in males is necessary to change drinking behavior and increase alcohol self-administration.

As a general trend, increases in alcohol self-administration were only observed on days where male or female imiquimod rats had decreased alcohol self-administration the day before. The decrease in alcohol self-administration on the day of injections coincides with days where rats had reduced locomotor activity and is likely an indication of a sickness response which is lowering the rats' motivation to work for alcohol. On days where no sickness response seems to have been induced (first injection in males), alcohol self-administration was not increased the following day. This suggests that the activation of an immune response is likely related to drinking behavior.

A 10 mg/kg dose of imiquimod was sufficient in activating an immune response in both males and females; however, there were differences between the patterns of immune response activation in either group. Decreased locomotor rate during an alcohol self-administration session is suggestive of an activated sickness response by the body's immune system. Following the same trend observed with alcohol self-administration, imiquimod females had reduced locomotor rates on the days of each injection starting on the initial injection, while imiquimod males had reduced locomotor rates on the days of injections two through four (Fig.3). Weight loss is another indication of an activated sickness response by the body's immune system (Kelley et al. 2003). Both imiquimod males and females experienced weight loss after injection days; however, normal weight gain resumed almost immediately the next day (Fig.4). The current experiments show that TLR7 activation induces a rapid (2 hour) immune response with acute sickness behavioral effects. Over time with repeated imiquimod injections, the spleens of both male and female imiquimod rats were significantly heavier compared to control rats (Fig.5). The

enlarged spleens of the imiquimod rats suggests that their immune system was hyperactive and that there was increased supply of immune cells being stored for defense.

Since we observed increased ethanol self-administration 24 hours after imiquimod injections, we wanted to understand what was happening genetically in the nucleus accumbens core at this 24 hour post-injection time point. Specifically, we were interested in assessing differences in expression of TLRs in rat AcbC due to evidence that there is a relationship between TLR activation and alcohol intake (Blednov et al. 2011). Our lab has previously found that activation of TLR3 resulted in increased expression of TLR7 in male rat Acb (Randall et al. 2019), which catalyzed our interest in targeting TLR7 activation. Our results indicate that repeated activation of TLR7 via imiquimod induces increased gene expression of TLR7 as well as TLR3. Both imiquimod males and females had significantly higher expression of TLR3 compared to control groups in the AcbC. Interestingly, only the imiquimod females had increased expression of TLR7 compared to the control group at this time point. The mechanism of TLR7 induced increases in ethanol self-administration and gene expression are complex. There seems to be a signaling relationship between TLR3 and TLR7 where activation of one results in the increased expression of the other. This is not unlikely due to the fact that TLR3 and TLR7 are both endosomal receptors that upon activation, trigger similar neuroimmune cascades (Nishiya et al. 2005).

An important consideration for the current experiments is that TLR7 escapes X chromosome inactivation, presumably making it more highly expressed in females than in males (Souyris et al. 2018). Differences in TLR7 expression could potentially be a variable affecting TLR7 sensitization in the males and females. Sex differences in immune activation has been well documented, with females generally mounting a stronger immune response than males (Marriot

and Huet-Hudson 2006; Schwartz and Bilbo 2012). There is evidence that females generally produce higher levels of proinflammatory cytokines than males (Aulock et al. 2006; Drew and Chavis 2000; Loram et al. 2012); and that following TLR activation specifically, females tend to show increased activation of proinflammatory responses (Klein and Flanagan 2016). The findings from the current experiments suggest that differences in immune function and activation likely contribute to the different patterns of drinking behavior observed in the male and female groups; however, future studies with larger sample sizes will allow us to have a more comprehensive understanding on whether these different patterns are sex-specific.

Taken together, the findings presented support a connection between TLR7 neuroimmune activation and ethanol self-administration as well as TLR gene expression. Numerous studies emphasize the important role that neuroimmune activation plays in the development of addiction (Coleman and Crews 2018) as well as the regulatory effects of the innate immune system on alcohol consumption (Crews et al. 2017a). Future experiments should assess how prolonged activation of TLR7, potentially by administering imiquimod injections multiple days in a row, would effect ethanol self-administration and gene expression of TLRs. Also, utilizing microinjections to administer imiquimod locally in the brain could provide a better understanding on how drinking behavior would be affected if the peripheral sickness response is eliminated. The findings presented support emerging evidence that neuroimmune activation can affect alcohol drinking behavior and likely contributes to the development of AUD. Future work to improve the understanding of which specific neurobiological factors of neuroimmune activation contribute to the pathology of AUD is critical for the development of enhanced treatment options.

Acknowledgements

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FIGURES:

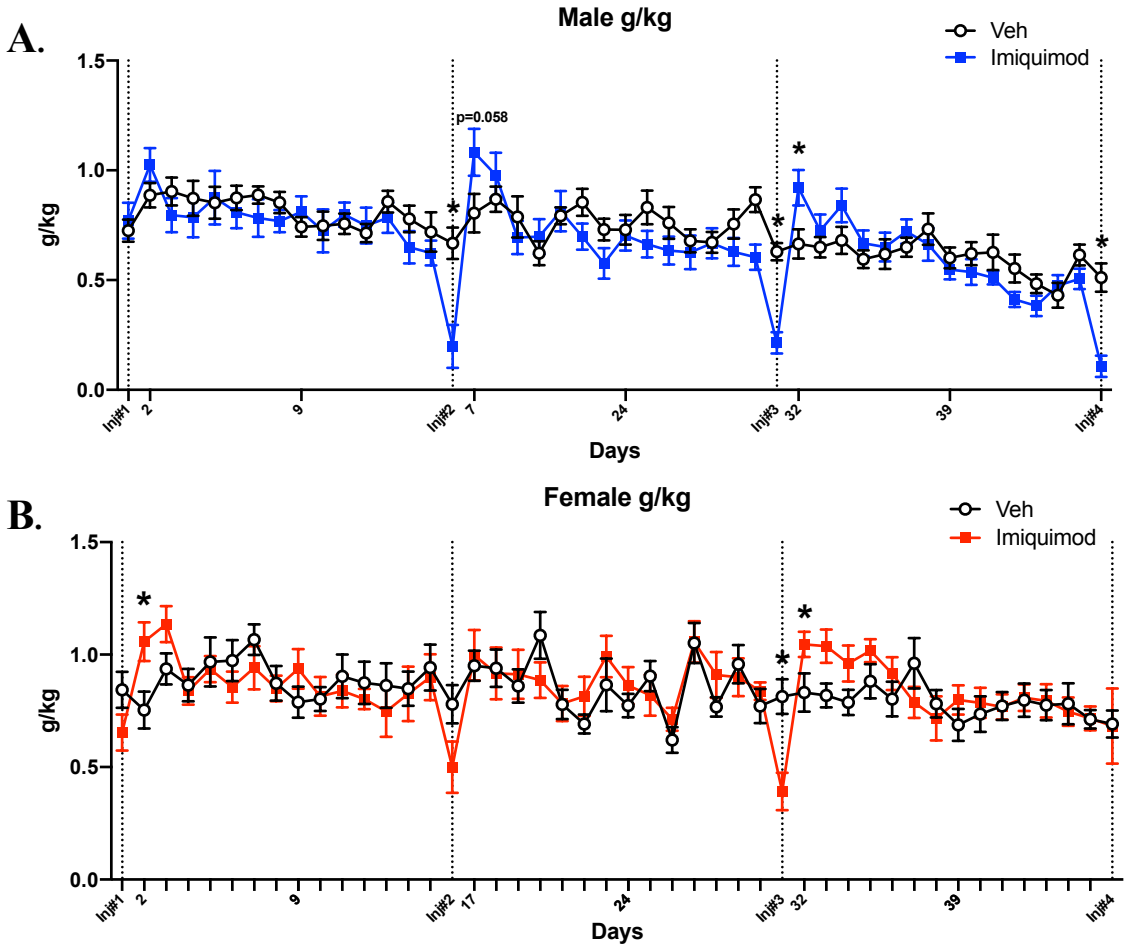

Figure 1.

Imiquimod increases alcohol intake (g/kg) in males and females. Male and female Long Evans rats received four imiquimod injections (10 mg/kg) over a 46-day span. Daily operant ethanol self-administration sessions were held to track changes in drinking behavior. Ethanol intake (g/kg) was calculated by dividing estimated grams of alcohol consumed by body weight. (A) Repeated imiquimod injections increased ethanol intake in the male imiquimod group. (B) The initial imiquimod injection increased ethanol intake in the female imiquimod group. The results are the means ± SEM. *=$p<0.05$

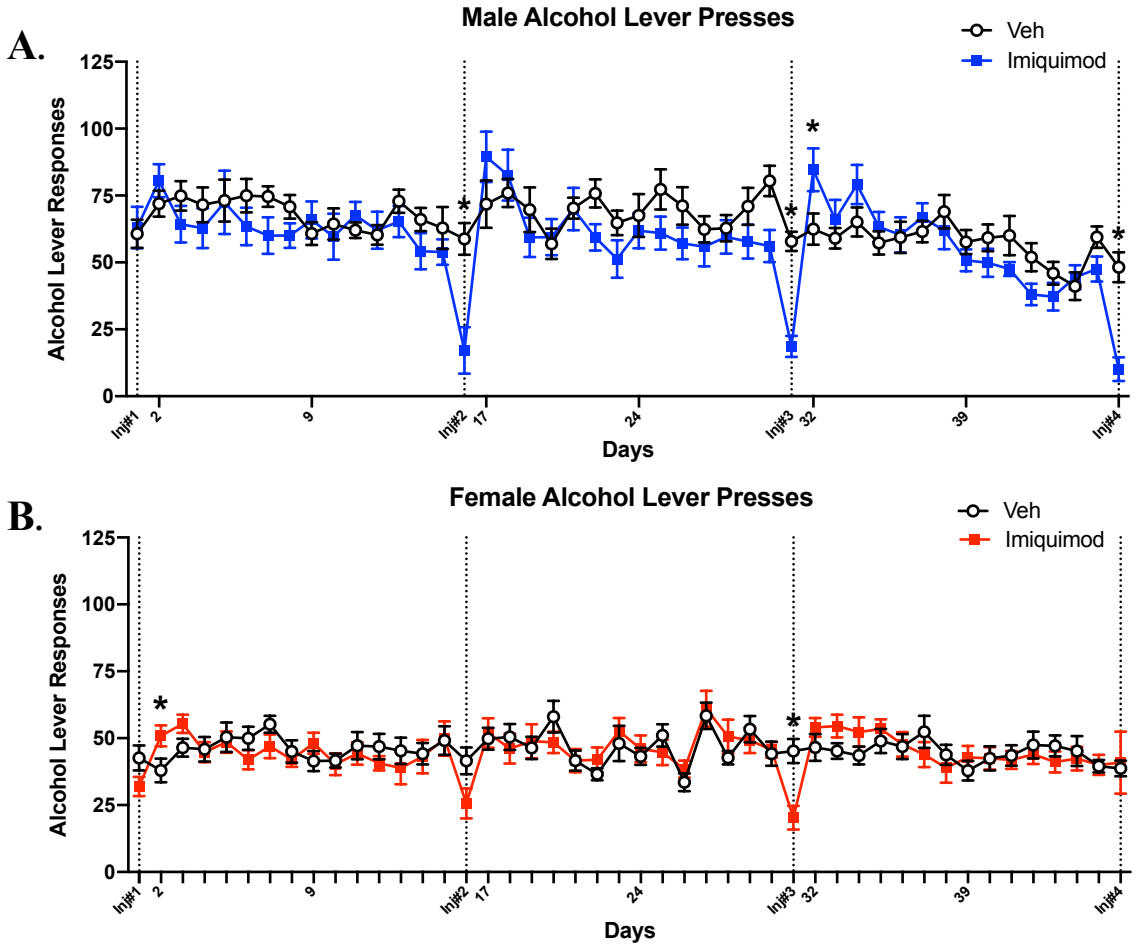

Figure 2.

Imiquimod increases alcohol lever presses in males and females. Male and female Long Evans rats received four imiquimod injections (10 mg/kg) over a 46-day span. Daily operant ethanol self-administration sessions were held to track changes in drinking behavior. Active lever presses count the number of times rats are pressing the alcohol lever which indicates their motivation for alcohol. (A) Repeated imiquimod injections increased alcohol lever presses in the male imiquimod group the day after the third injection. (B) In females, the initial imiquimod injection increased alcohol lever presses 24 hours later in the female imiquimod group while subsequent injections did not. Data are means ± SEM. *=$p<0.05$

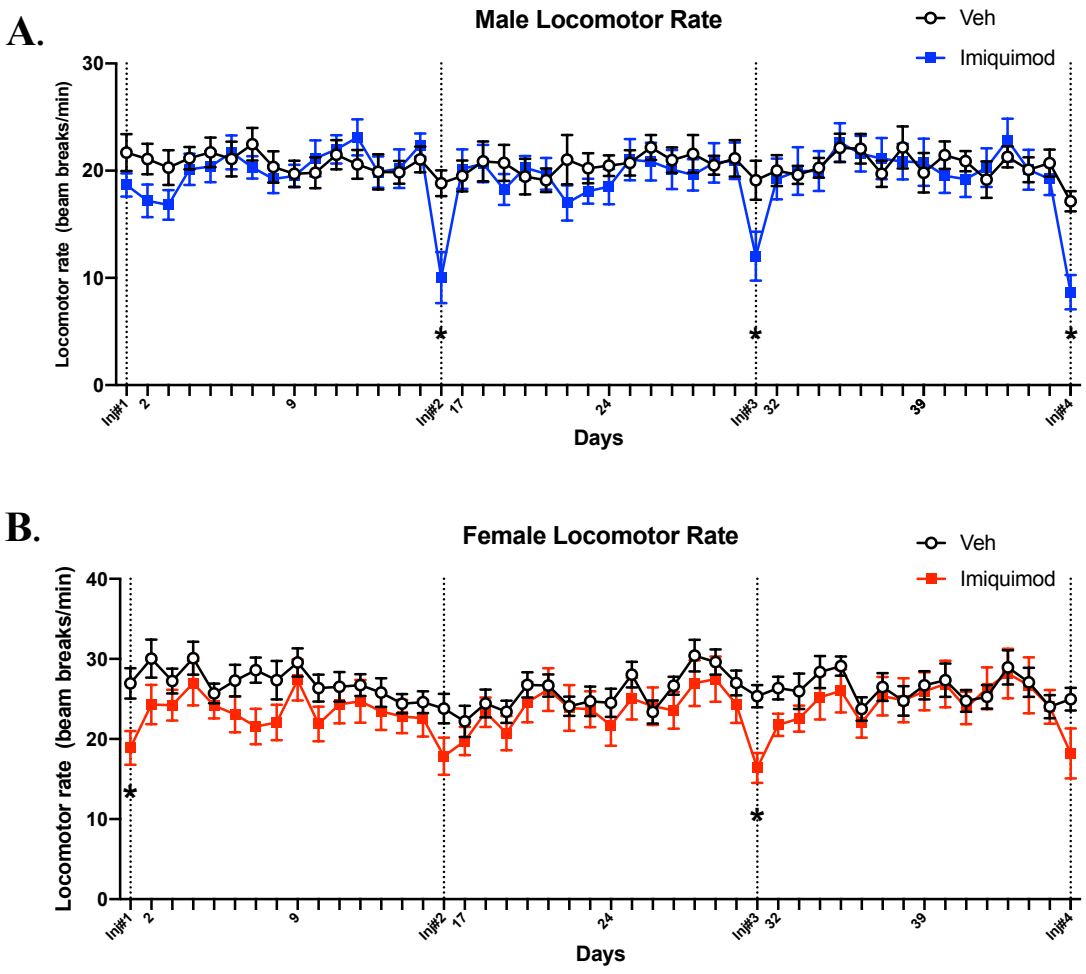

Figure 3.

Reduced activity on days of imiquimod injections. Locomotor rate, calculated by number of beam breaks during operant chamber sessions, assesses total activity on a given day. (A) Male imiquimod rats had reduced activity two hours after each injection other than the first injection. (B) Female imiquimod rats had reduced activity two hours after the first injection and similar results were observed with each subsequent injection. Data are means ± SEM. *=$p<0.05$

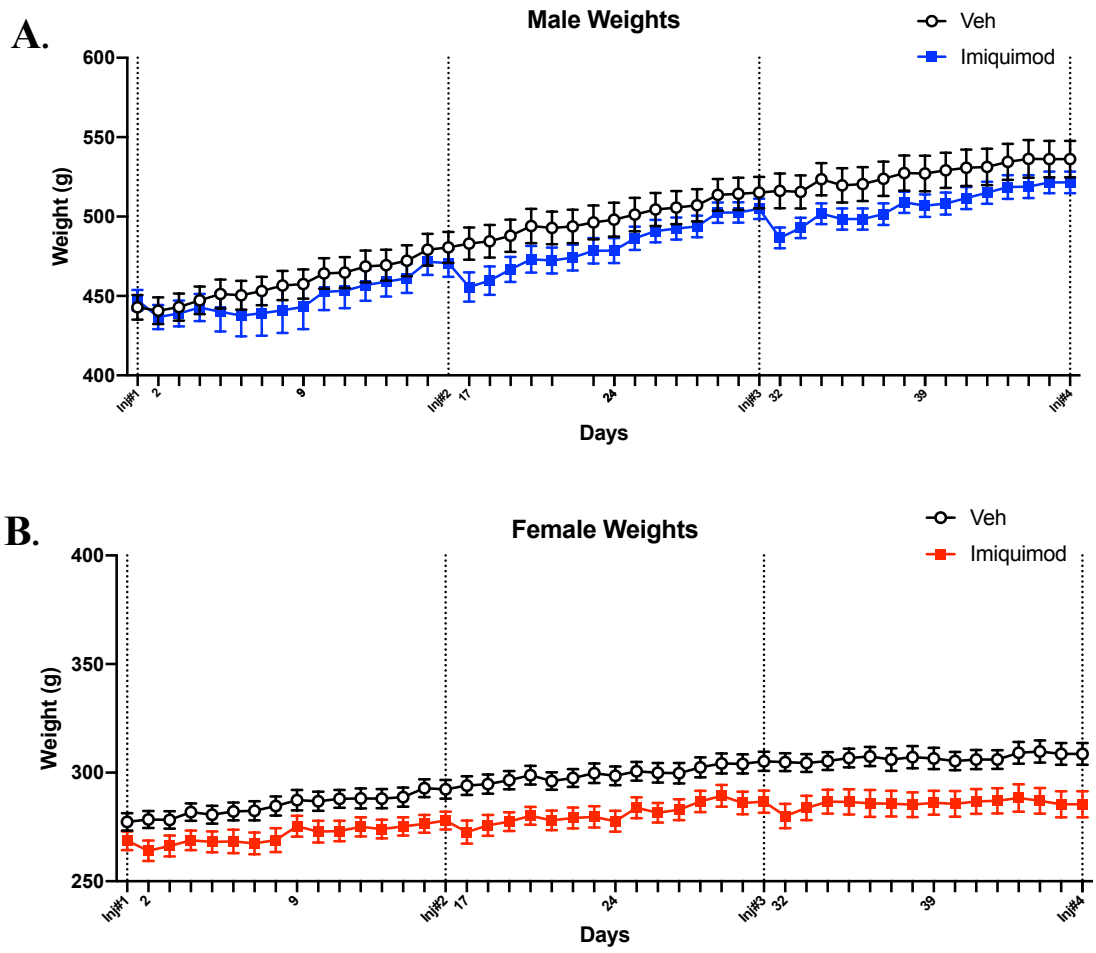

Figure 4.

Weight loss in imiquimod groups the day after injections. Body weights were measured daily immediately before self-administration sessions. Weight loss was observed the day following injections in both imiquimod males (A) and females (B). Normal weight gain resumed the following day. The results are the means ± SEM.

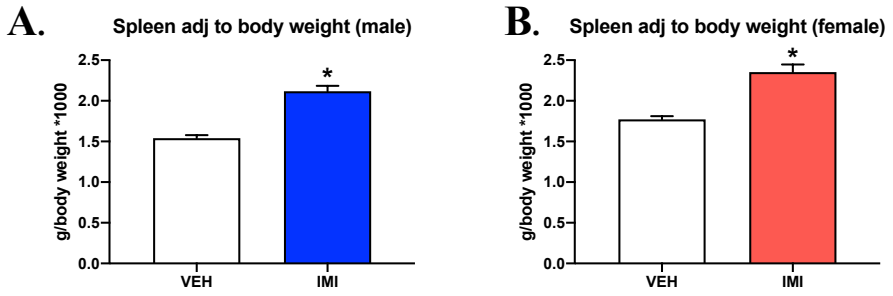

Figure 5.

Effect of imiquimod injections on spleen weight. Spleens were collected and weighed 24 hours after the fourth imiquimod injection. Spleen weights were adjusted to account for differences in body weight. Both imiquimod males (A) and females (B) had significantly heavier spleens compared to control groups. The results are the means ± SEM. *=p<0.05

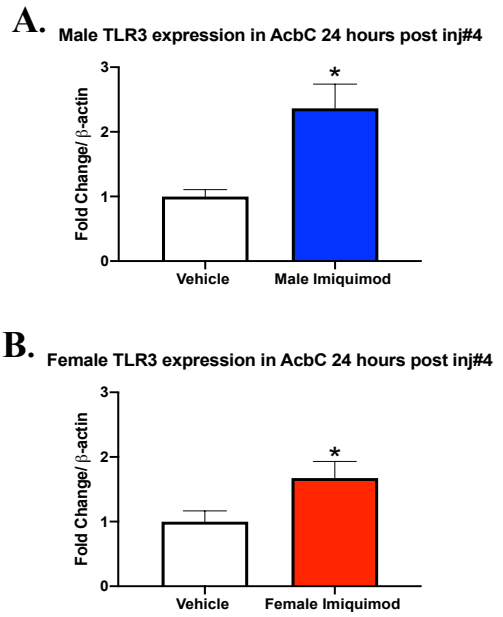

Figure 6.

Increased TLR3 expression in male and female rat AcbC. Repeated imiquimod injections increased mRNA expression of TLR3 in imiquimod male (A) and female (B) AcbC 24-hours after the fourth injection. The results are the means ± SEM. *=p<0.05

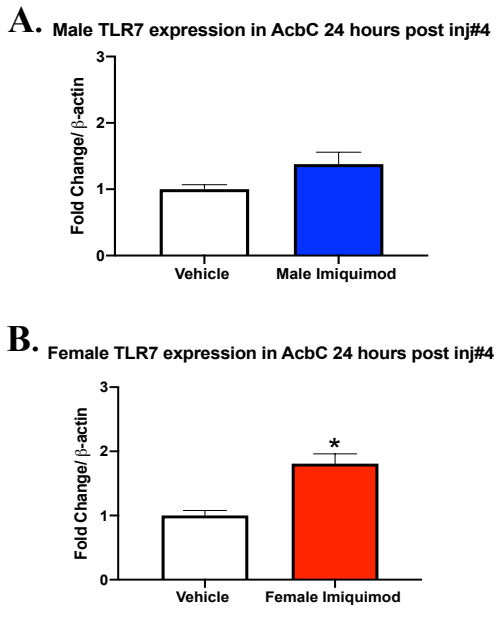

Figure 7.

Increased TLR7 expression in female rat AcbC. (A) Repeated imiquimod injections had no effect on mRNA expression of TLR7 in males. (B) Imiquimod females had increased TLR7 mRNA expression in the AcbC 24 hours after the fourth injection. The results are the means ± SEM.